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Prediction of Intracellular Amount of 1-Methyladenine Precursor in Ovarian Follicle Cells of the Starfish, *Asterina pectinifera*

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ABSTRACT—Resumption of meiosis in starfish oocytes is induced by 1-methyladenine (1-MeAde) produced by overian follicle cells under the influence of gonad-stimulating substance (GSS). The present study was undertaken to determine whether 1-MeAde production in follicle cells following stimulation by GSS is due to the release of stored 1-MeAde or to *de novo* synthesis using a precursor of 1-MeAde. Although 1-MeAde produced by follicle cells in *Asterina pectinifera* was found in the extracellular medium following incubation with GSS, 1-MeAde did not preexist in these cells. Also, the continual presence of GSS in the medium did not maintain 1-MeAde production: as incubation time was prolonged, the level of 1-MeAde production gradually declined and finally stopped. Although 1-MeAde production had already ceased in follicle cells after incubation for 12 hr with GSS, GSS still caused an increase in the intracellular levels of cyclic AMP. It has also been reported that methionine and selenomethionine enhance the GSS-induced 1-MeAde production. In this study it was found that the total amounts of 1-MeAde produced by follicle cells were almost the same regardless of whether methionine or selenomethionine was present. Each follicle cell was capable of producing about 1 fmol 1-MeAde. These results strongly suggested that 1-MeAde is newly synthesized using a precursor stored in follicle cells.

INTRODUCTION

In most animals, meiosis in fully grown oocytes is arrested at the prophase of the first maturation division. Hormonal control is required for the resumption of meiosis. In starfish, resumption of maturation division is triggered by 1-methyladenine (1-MeAde) [1–4], which is known to be produced in follicle cells by the action of a gonad-stimulating substance (GSS) secreted from the neural system [5, 6]. A previous *in vitro* study using follicle cells of *Asterina pectinifera* has demonstrated that 1-MeAde produced under the influence of GSS is not a breakdown product of some 1-MeAde-containing substance such as ribonucleic acid, but is synthesized *de novo* [7]. The role of GSS in the production of 1-MeAde in *A. pectinifera* and *Asterias amurensis* has also been shown to involve activation of the transfer of a methyl group to the N_1 site of the purine nucleus of a 1-MeAde precursor [8, 9]. In contrast, it was reported recently that 1-MeAde production in *Pisaster ochraceus* is due to the release of intracellular 1-MeAde stored in follicle cells [10]. To elucidate whether 1-MeAde preexists in the follicle cells of *A. pectinifera* before GSS stimulation, the present study was undertaken to determine the intracellular 1-MeAde content.

It was shown recently that upon incubation of starfish follicle cells with GSS there is a dose-related increase in cyclic adenosine 3′,5′-monophosphate (cAMP) production, coincident with an increase in 1-MeAde production [11–13]. With respect to the increase in cAMP levels, GSS causes activation of the adenylate cyclase system involving guanine-nucleotide regulatory binding proteins [14]. These results suggest that cAMP plays an important role in mediating the action of GSS on 1-MeAde production, although the regulatory mechanism of 1-MeAde production by cAMP is poorly understood. This study also investigated

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the role of cAMP in 1-MeAde production by starfish follicle cells.

MATERIALS AND METHODS

Animals

Starfish, *Asterina pectinifera,* were collected at Hashirimizu (Kanagawa, Japan) and Asamushi (Aomori, Japan). The animals were kept in laboratory aquaria with circulating artificial sea water, 'My Sea' (Jamarin Laboratory, Osaka, Japan) at 15°C.

Reagents

1-MeAde and selenomethionine were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were of analytical grade.

The sea water was modified van't Hoff's artificial sea water (ASW) adjusted to pH 8.2 with 0.02 M borate buffer [15]. GSS was prepared from the lyophilized radial nerves of *A. pectinifera* as previously described by Kanatani *et al.* [16] and Shirai [17]. The amount of GSS was expressed as the original nerve weight (dry nerve weight equivalent).

Preparation of follicle cells

Follicle cells were isolated as described previously [12, 18]. The number of follicle cells was estimated from the number of oocytes, since each oocyte is enclosed by approximately fifty follicle cells (50 ± 4 : mean ± SEM of nine separate estimations).

Incubation of follicle cells

One million follicle cells isolated from 2×10^4 oocytes were incubated at 20°C with occasional shaking in 1.0 ml ASW containing GSS at a concentration of 0.1 mg nerve equivalent/ml. The detailed protocol of the experiment will be given under Results. After incubation, the cell suspension was centrifuged at $1{,}000 \times g$ for 1 min and the supernatant assayed for 1-MeAde. The concentration of 1-MeAde was determined by a method described previously [17, 19], using authentic 1-MeAde as a standard reference. The amount of 1-MeAde was expressed in nmol/ml. The sedimented cells were quickly frozen in dry ice-acetone and used for determination of cAMP. The cAMP was measured as described previously [20] using a commercial radioimmunoassay kit (Yamasa Shoyu Company, Chiba, Japan), following extraction of the cells with 6% trichloroacetic acid (TCA).

Measurement of intracellular 1-MeAde content

Before and after incubation of 10^6 follicle cells with GSS at a concentration of 0.1 mg nerve equivalent/ml for 2 hr at 20°C, the cells were washed twice with ASW and homogenized with 1.0 ml 6% TCA containing authentic 1-MeAde at a desired concentration. The homogenate was then centrifuged at $5{,}000 \times g$ for 5 min. The supernatant was washed three times with water-saturated diethylether, and the aqueous phase collected and lyophilized. The samples were dissolved serially in ASW in a total volume of 1.0 ml and assayed for 1-MeAde.

RESULTS

When isolated follicle cells ($1.0 \pm 0.1 \times 10^6$ cells) from 2×10^4 oocytes were incubated for 2 hr with ASW containing GSS at a concentration of 0.1 mg nerve equivalent/ml, about 0.47 nmol 1-MeAde produced was found in 1.0 ml of incubation medium. An experiment was carried out to determine if 1-MeAde preexists in follicle cells before GSS treatment. One million follicle cells were homogenized with 6% TCA containing authentic 1-MeAde at a concentration of 0.125, 0.25, 0.5 or 1.0 μM. After removal of TCA by diethylether partition, these extracts were used for 1-MeAde determination. As shown in Figure 1, the added 1-MeAde was completely recovered from the cell extracts. However, 1-MeAde could not be detected in the extract without added 1-MeAde. A similar standard curve was obtained from the extracts of follicle cells incubated for 2 hr with GSS at a concentration of 0.1 mg nerve equivalent/ml. It was shown clearly that neither non-GSS-treated nor treated follicle cells contained 1-MeAde.

However, during incubation with the same quantity of GSS, the amount of 1-MeAde produced by follicle cells increased in the extracellular

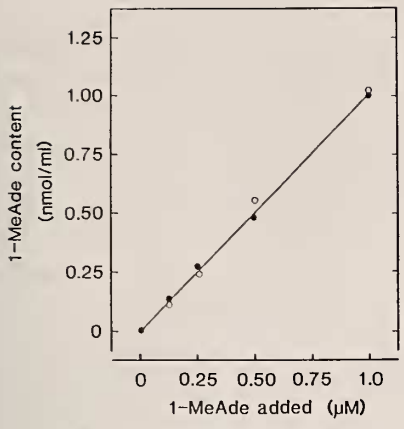

FIG. 1. Intracellular concentration of 1-MeAde in follicle cells of *A. pectinifera*. Before (●) and after (○) incubation with GSS (0.1 mg nerve equivalent/ml) for 2 hr at 20°C, 10^6 follicle cells were washed with ASW twice, and suspended and homogenized in 1.0 ml 6% TCA containing 1-MeAde at the indicated concentrations. After centrifugation, the supernatant was partitioned with water-saturated diethylether to remove TCA, and the aqueous phase was collected and lyophilized. The sample was dissolved in 1.0 ml ASW and assayed for 1-MeAde.

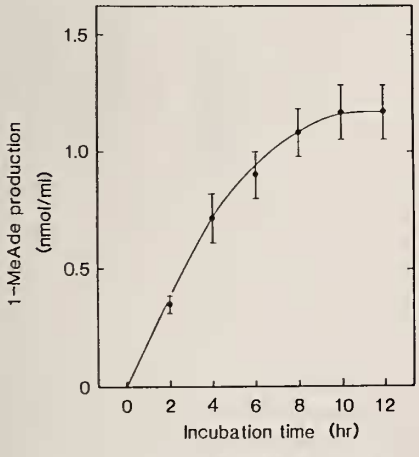

FIG. 2. Effect of GSS on 1-MeAde production in follicle cells. One million follicle cells were incubated with 1.0 ml ASW containing GSS (0.1 mg nerve equivalent/ml) at 20°C. After incubation for the indicated times, the cell suspension was centrifuged and the supernatant assayed for 1-MeAde. Each point represents the mean ± SEM of three separate experiments.

medium (Fig. 2). The continual presence of GSS in the medium induced almost linear 1-MeAde production at least for the first 4 hr. As the incubation time was prolonged, the rate of 1-MeAde production gradually declined. At 10 hr, the total amount of 1-MeAde produced by follicle cells reached a plateau. After incubation of 10^6 follicle cells for 12 hr with GSS, 1.2 ± 0.1 nmol 1-MeAde was accumulated in 1.0 ml of medium. Therefore, it was calculated that about 1 fmol 1-MeAde was produced by a follicle cell.

The cessation of 1-MeAde production by follicle cells upon prolonged incubation might have been due to loss of GSS activity and/or cell death. This possibility was tested by preincubation of follicle cells with ASW in the absence and presence of GSS, before incubation with fresh GSS. When follicle cells were preincubated with GSS at a concentration of 0.1 mg nerve equivalent/ml, the amount of 1-MeAde produced during a further 2-hr incubation with fresh GSS decreased markedly as the preincubation time was prolonged (Fig. 3). After 12-hr preincubation with GSS, the follicle cells failed to produce 1-MeAde under the influence of fresh GSS. In contrast, when follicle cells were preincubated for 2, 6 or 12 hr without GSS, 1-MeAde production by follicle cells occurred upon addition of GSS. Amounts of 1-MeAde

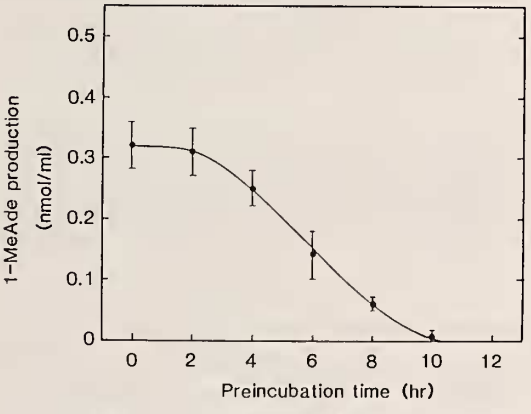

FIG. 3. Effect of preincubation with GSS on 1-MeAde production in follicle cells. One million follicle cells were preincubated with 1.0 ml ASW containing GSS (0.1 mg nerve equivalent/ml) at 20°C. After preincubation for the indicated times, the cells were washed twice with ASW and resuspended in 1.0 ml ASW containing fresh GSS (0.1 mg nerve equivalent/ml). Incubation was carried out for 2 hr at 20°C. After centrifugation, the supernatant was assayed for 1-MeAde. Each point represents the mean ± SEM of three separate experiments.

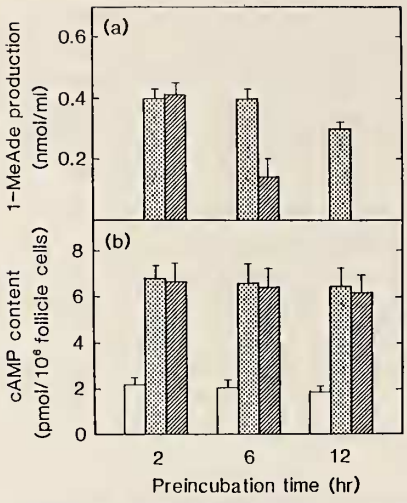

FIG. 4. Effects of preincubation time on 1-MeAde (a) and cAMP (b) production induced by GSS in follicle cells. One million follicle cells were preincubated with 1.0 ml ASW at 20°C in the absence (▒) or presence of GSS (0.1 mg nerve equivalent/ml) (▨). After preincubation for 2, 6 or 12 hr, the cells were washed twice with ASW and resuspended in 1.0 ml ASW containing fresh GSS (0.1 mg nerve equivalent/ml). Incubation was then carried out for another 2 hr at 20°C. After centrifugation, the supernatant was assayed for 1-MeAde, and the sedimented cells were used for determination of cAMP. Each point represents the mean ± SEM of three separate experiments. (□), Levels in control experiments, involving incubation without GSS.

similar to those without preincubation were produced by the follicle cells (Fig. 4a). These results indicated that prolonged incubation with ASW alone did not prevent follicle cells from producing 1-MeAde.

Previous studies have shown that following incubation with GSS, intracellular cAMP levels are increased, coincident with an increase in 1-MeAde production [11–13]. In the next experiment, the effect of GSS on cAMP production was examined during prolonged incubation. After preincubation of 10^6 follicle cells for 2, 6 or 12 hr with or without GSS at a concentration of 0.1 mg nerve equivalent/ml, they were washed and reincubated with the same quantity of GSS. cAMP levels in these follicle cells increased in response to fresh GSS (Fig. 4b).

The present study had already shown that 1-MeAde production by follicle cells was not unlimited (Fig. 2). It was therefore of interest to determine whether the total amount of 1-MeAde produced by follicle cells depended on the size of an intracellular pool of 1-MeAde precursor. A previous study showed that the role of GSS in 1-MeAde production was stimulation of methylation [8]. It has also been shown that GSS-induced 1-MeAde production is enhanced by methionine [8, 9] and selenomethionine [21]. These findings suggest that 1-MeAde is synthesized from an unknown precursor through methylation. As shown previously [8, 9, 21], during the first 2 hr of incubation, methionine (5 mM) and selenomethionine

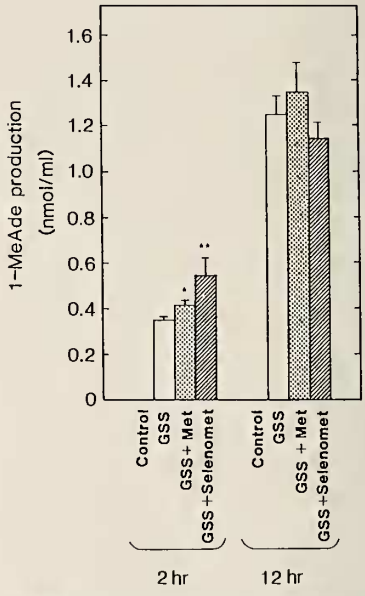

FIG. 5. Effects of methionine and selenomethionine on 1-MeAde production induced by GSS in follicle cells. After 10^6 follicle cells had been preincubed for 30 min with ASW at 20°C in the absence (□) or presence of either methionine (5 mM) (▒) or selenomethionine (0.5 mM) (▨), incubation was initiated by addition of GSS (0.1 mg nerve equivalent /ml). At 2 and 12 hr, the cell suspension was centrifuged and the supernatant assayed for 1-MeAde. Each point represents the mean ± SEM of three separate experiments. Levels of 1-MeAde production in control experiments, in which follicle cells were incubated without GSS, were not detectable. *P* values were calculated using Student's *t* test and compared with the results obtained with GSS alone: *$P<0.1$, **$P<0.05$.

(0.5 mM) significantly increased the level of 1-MeAde production induced by GSS (0.1 mg nerve equivalent/ml) (Fig. 5). Despite this, addition of either methionine or selenomethionine did not alter the total amounts of 1-MeAde produced by follicle cells after incubation for 12 hr. Similarly, the total amount of 1-MeAde produced by 10^6 follicle cells was about 1.2 nmol/ml.

DISCUSSION

The present results provide further evidence to support the previous proposal [7] that 1-MeAde is synthesized *de novo* in follicle cells of *A. pectinifera* under the influence of GSS. The role of GSS in 1-MeAde production is not merely induction of the release of intracellular 1-MeAde stored in follicle cells. The present study also showed that 1-MeAde production by follicle cells does not continue indefinitely even in the presence of GSS. After prolonged incubation with GSS, follicle cells stopped producing 1-MeAde. The induction of 1-MeAde production by GSS is mediated through elevation of the second messenger cAMP [11–13]. In contrast, after preincubation for a long period with GSS, an increase in intracellular cAMP levels was found following further incubation with GSS. These results indicate that upon prolonged incubation with GSS, although follicle cells still retain their ability to respond to GSS, cAMP fails to induce 1-MeAde synthesis. It seems that the failure of 1-MeAde production is due to exhaustion of 1-MeAde precursors during incubation.

Exogenous 1-methyladenosine (1-MeAde-R) and 1-methyladenosine monophosphate (1-MeAMP) have been shown to be as effective as GSS in evoking 1-MeAde production [22, 23]. 1-MeAde-R and 1-MeAMP seem to be precursors of 1-MeAde. However, follicle cells possess activity of 1-MeAde-R ribohydrolase, the enzyme that converts 1-MeAde-R to 1-MeAde [24, 25]. If 1-MeAde-R and 1-MeAMP preexist in follicle cells, they should be converted into 1-MeAde. Thus, 1-MeAde-R and 1-MeAMP appear not to be stored in follicle cells, even if they are direct precursors of 1-MeAde. These compounds may, in fact, serve not as direct precursors but as intermediates in 1-MeAde biosynthesis.

When follicle cells were incubated with GSS in the presence of either methionine or selenomethionine, the extracellular levels of 1-MeAde during first 2 hr of incubation were higher than with GSS alone. This result is in accord with an earlier suggestion that a methylation process is involved in GSS-dependent 1-MeAde biosynthesis [8, 9, 21]. The present study also showed that, during 12 hr of incubation, the total amounts of 1-MeAde produced in the presence of GSS plus methionine or selenomethionine are comparable to the values obtained with GSS alone. These results suggest that the amount of 1-MeAde produced by follicle cells may depend on the pool size of a precursor of 1-MeAde as an acceptor of methyl radicals. If so, the concentration of this unknown 1-MeAde precursor stored in follicle cells is almost equal to that of 1-MeAde produced. Since a limited amount of 1-MeAde production, about 1 fmol 1-MeAde per follicle cell, was measured precisely in the present study, the same quantity of precursor may be stored in each cell. The identify of this precursor of 1-MeAde is currently under investigation.

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